Determination of Amide Nitrogen in Collagen and Other Proteins*

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ABSTRACT

An alkaline hydrolysis method for the determination of amide nitrogen is described. Values obtained by this method compare favorably with the literature values for a number of proteins. A study of the amide nitrogen content of collagen obtained from the hides of identical twin cattle subjected to different nutritional levels shows that the amide value of the collagen is not affected in these experiments. Some changes in amide values of collagen and gelatin due to processing are indicated.

INTRODUCTION

Amide nitrogen is generally determined by the acid hydrolysis methods of either Shore et al.[1] or Gordon et al.[2]. These methods trap the liberated ammonia in the hydrolyzing acid from which it must be liberated by strong alkali and distilled into a medium in which it may be estimated by titration. The kinetics of this method have recently been studied by Cassel and MacKenna[3]. Warner and Cannan[4] proposed an alkaline hydrolysis method which was carried out in a Conway vessel[5]. The liberated ammonia diffused from the hydrolysis medium to the standard acid titrating medium which was enclosed within the same vessel.

These three methods require a considerable amount of work to demonstrate that all of the amide nitrogen has been liberated from the sample because a separate sample must be run for each point on the time curve. The present paper reports an adaptation of the Warner method wherein the procedure is simplified by using a train of Sobel microaeration tubes[6] instead of the Conway vessels.

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Details of Method

The determination is carried out in a train of Sobel microaeration tubes as shown in Figure 1. The first tube which has the bubbling side arm open to

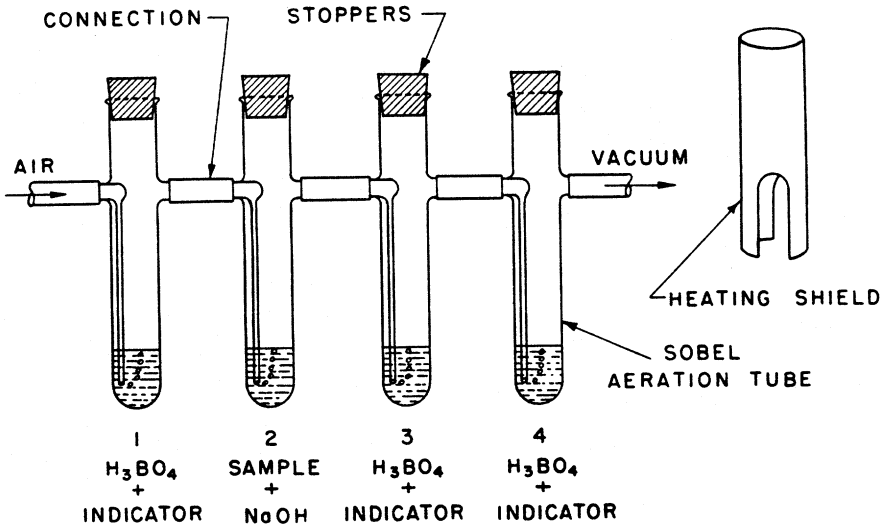

FIGURE 1. Amide Nitrogen Apparatus.

the atmosphere contains 1.5 ml. of boric acid-indicator solution. This tube prevents atmospheric ammonia from influencing the results. The second tube contains the sample, which should contain about 0.3 mg. of amide nitrogen, 3 drops of silicone antifoam solution, and 2 ml. of 2.0 *N* sodium hydroxide solution which was added at the start of the determination. The third and fourth tubes each contain 1.5 ml. of boric acid-indicator solution.

The tubes are connected with 1.25 inch long sections of $^3/_{16}$ inch bore rubber tubing so that the bubbling side arm of each tube is connected to the previous tube. These connections are made and tubes 1, 3 and 4 are stoppered with rubber stoppers before the alkali is added to tube 2. The fourth tube is connected to a vacuum source through a flow regulating device so that an air flow of about 120 bubbles per minute can be maintained throughout the determination. A very fine capillary several inches in length has been found most suitable for producing the desired regulation. The ends of the capillary must be protected from dust by glass wool or other plugs. The vacuum should be connected just prior to introducing the alkali to tube 2 and tube 2 should be stoppered immediately after the alkali is introduced.

The tubes are then set into a rack which straddles the side of a 70° water bath so that the first two tubes (the pretrap and reactor) are immersed in the bath and the last two tubes (the absorbers) are hanging in air at room temperature. The reactor tube is then covered with a section of slotted copper tubing which extends from the top of the reactor tube down to one-half inch below the water level of the bath. This tube conducts heat from the bath to the top of the reactor and prevents condensation of moisture and adsorption of ammonia in this portion of the reactor tube.

At the desired time intervals (2, 3, 4, 5 and 6 hours after starting the determination have been found suitable) the air flow is stopped, the first absorption tube disconnected and the tubing from the reactor tube closed with a pinchcock. The closing of the reactor tube is necessary to prevent the loss by diffusion of any ammonia remaining in the connector tube. The ammonia

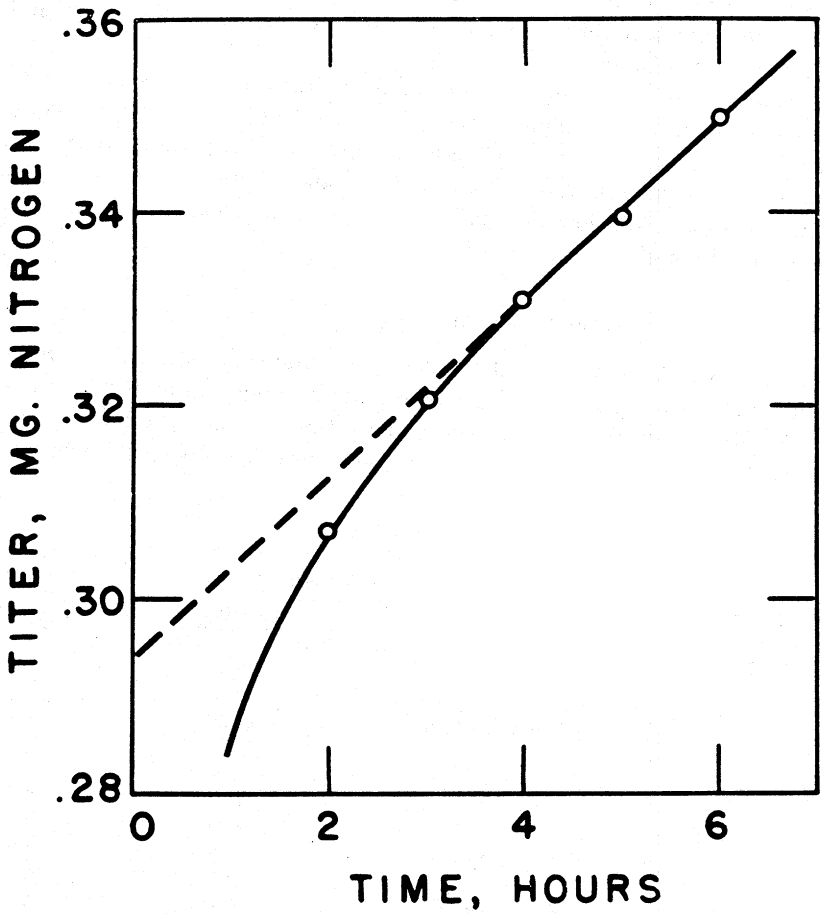

FIGURE 2. The rate of Ammonia liberation.

trapped in the absorption tube is then titrated with 0.1 *N* hydrochloric acid. A one ml. capacity microburette is used for this titration. A blank tube containing only the boric acid-indicator solution may be used to match the indicator end point color where the highest accuracy is desired. The indicator color in the second absorption tube usually will not change. However, should there be a change in color this tube must be titrated also. The absorption tubes are returned to the train and the air flow resumed. The accumulated titers are plotted against the times (Figure 2) and the straight line portion of the curve is extrapolated to zero time to give the titer due to the amide nitrogen liberated.

Special Reagents

Sobel's mixed indicator—8 parts of a 0.1 per cent solution of bromocresol green in 95 per cent alcohol and 1 part of a 0.1 per cent solution of methyl red in 95 per cent alcohol.

Boric acid-indicator solution—2.0 gm. of boric acid crystals and a 1 ml. of Sobel's mixed indicator are made to 100 ml. with water. The indicator color is adjusted to a very faint purple with a few drops of 0.01 *N* sodium hydroxide when the volume is within about 2 ml. of the final volume.

Silicone anti-foam solution—is made by suspending about one-half gram of silicone anti-foam-A in 100 ml. of ether.

Experimental Verification

The method was employed to determine the amide nitrogen of a number of proteins including casein, edestin, β-lactoglobulin, ovalbumin and serumalbumin. All of these were of the purest grade available commercially for laboratory work. Table I shows that the results obtained with this proposed

TABLE I

The Amide Nitrogen Content of Various Proteins

Protein	Determined % of total N	Literature % of total Nitrogen	Reference
Casein	10.7 ± 0.14 (8)*	10.2	7
		9.1	8
Edestin	10.7 ± 0.15 (17)	10.3	4
		9.5	8
β–lactoglobulin	6.8 ± 0.21 (12)	6.9	4
		6.9	8
		6.9	9
Ovalbumin	7.0 ± 0.22 (11)	6.2	1
		6.6	8
Bovine Serum Albumin	5.8 ± 0.17 (6)	5.6	9

*The standard deviation calculated for the number of determinations given in the brackets.

method agree very well with previous values reported in the literature. A standard error of around 0.2 was obtained for each protein where sufficient experimental results had been collected to enable the calculation of the standard error. The method, therefore, seems to be suitable for determining or following changes in amide content of proteins in general.

Collagen and Gelatin Studies

The method was then applied to determine the amide nitrogen content of a number of collagen and gelatin preparations. The results are shown in Table II. In general the amide values center around 3.6 per cent of the total nitrogen. The three major exceptions are the American standard hide powder

TABLE II

The Amide Nitrogen Content of Various Collagen and Gelatin Preparations

Sample	Description	% Total Nitrogen	% of N as Amide
1	American std. hide powder	17.87	2.41
2	Limed "white" hide	17.63	2.61
3	Purified collagen (Cassel)	18.05	3.93
4	Defatted salted hide	16.90	3.79
22	Twin hides—normal diet	17.83	3.72
21	—restricted diet	18.05	3.80
19	Twin hides—normal diet	18.20	3.28
20	—restricted diet	18.03	3.41
31	Twin hides—normal diet	18.28	3.64
32	—restricted diet	18.13	3.66
8	Purified collagen	17.93	3.72
8a	8-Ether alcohol extd.	18.22	3.54
8b	Gelatin from 8a	18.61	3.17
9	Eastman pig skin gelatin	17.82	3.55
10	Eastman calf skin gelatin	17.15	0.42

which gives a value of 2.4 per cent, limed hide (in the white from a commercial tannery), which gives a value of 2.6 per cent, and the Eastman calf skin gelatin with an isoelectric point of 4.7, which gives a value of 0.42 per cent.

Sample 3 which was prepared by Dr. J. M. Cassel as a purified collagen has the highest amide value. The comparatively mild treatment received by this sample during its preparation would indicate that the amide value of 3.9 per cent of the total nitrogen should be close to the maximum value to be expected for collagen. Cassel and Kanagy[10] have proposed the value of 3.8 per cent in their specification for purified collagen. The amide value for a sample (No. 4) taken from a defatted salted hide also had a value of 3.8 per cent.

The values for the standard hide powder and the limed "white" hide are in close agreement and the difference between these values and those of the purified collagen figures is probably due mainly to the liming treatment to which each of these materials had been subjected during their preparation.

Samples 19, 20, 21, 22, 31 and 32 are from the center split of the bend portions of hides taken from three pair of identical (monozygotic) twin cattle which had been subjected to nutritional studies [11], [12]. One twin was kept on a standard diet and sacrificed at a given weight. The other twin was put on a substantially reduced diet (deficient only in calories) for about 90 days and then fed to regain the lost weight and was sacrificed at an equal degree of fatness as the control twin. The sample numbers of the hides in Table II correspond to the animal numbers in reference 12 where details of the feeding experiments are described. These three pairs of hides were salted and preserved until the time they were worked up into the purified collagen. The six hides were extracted simultaneously in the same tumbler so that all treatments could be called comparable. They were washed with water and 8 per cent salt solution, fleshed, clipped and split to obtain the center layer. The center layer was then extracted 3 times each with 8 per cent salt, acetone, water, half saturated lime, water, dilute acetic acid, water and acetone. The samples were then air dried and ground in a Wiley mill. The close agreement of both the total nitrogen and the amide values for the collagen from the hide of each pair of twin animals would indicate that the reduced diet did not result in the production of an altered collagen. This is in agreement with the study performed by Lightfoot and Coolidge [13] on guinea pigs. They concluded that collagen continues to be produced in growing animals at the same rate in fasting animals as in well fed animals and that collagen formation is quantitatively the most important bioligical reaction using nitrogen for growth.

The slight variations in the amide values between the pairs of twins might be due to a number of factors but the data are not adequate to permit a proper choice among them.

The magnitude of the changes in amide values produced by fairly mild treatments is shown in the samples numbered 8. The original collagen (No. 8) was prepared from a fresh hide by extraction with 10 per cent salt solution, acetone, half saturated lime water, and dilute acetic acid. It gives nitrogen and amide values close to those of the best purified collagens. Extraction of this collagen with ether and warm alcohol followed by water (8a) has reduced the amide value by 0.2 units. Extraction of this ether extracted collagen with water at pH 7.0 and 120°C. for 30 minutes produced a gelatin (8b) which had an amide value 0.3 unit lower or 0.5 units lower than the original collagen.

The two commercial purified gelatins (samples 9 and 10) show the wide variation of amide values which it is possible to obtain without too much degradation to the gelatin molecule.

These examples show that the alkaline hydrolysis method for determining amide content of proteins can be carried out in a simple fashion to obtain values comparable with those in the literature. When the method was applied to a study of the purified collagens obtained from a series of identical twin cattle subjected to nutritional experiments the data showed that the amide content of the collagen did not depend upon the nutritional history of the animal if the animal was in a satisfactory nutritional state when sacrificed.

References

1. Shore, A., Wilson, H., and Stueck, G., *J. Biol. Chem.* **112,** 407 (1935).
2. Gordon, A. H., Martin, A.J.P., and Synge, R.L.M., *Biochem. J.* **35,** 1369 (1941).
3. Cassel, J. M., and McKenna, E., *J. Am. Leather Chem. Assoc.* **48,** 142 (1953).
4. Warner, R. C., and Cannan, R. K., *J. Biol. Chem.* **142,** 725 (1942).
5. Conway, E. J., and Byrne, A., *Biochem. J.* **27,** 419 (1933).
6. Sobel, A. E., Hirschman, A., and Besman, L., *Anal. Chem.* **19,** 927 (1947).
7. Gordon, W. G., Semmett, W. F., Cable, R. S., and Morris, M., *J. Am. Chem. Soc.* **71,** 3293 (1949).
8. Rees, M. W., *Biochem. J.* **40,** 632 (1946).
9. Brand, E., *Ann. N. Y. Acad. Sci.* **47,** 187 (1946).
10. Cassel, J. M., and Kanagy, J. R., *J. Res. Nat. Bur. Std.* **42,** 557 (1949).
11. Winchester, C. F., *Science* **116,** No. 3002, 3 (1952).
12. Winchester, C. F., and Hendricks, W. A., *U. S. Dept. Agriculture Technical Bulletin* **No. 1071** (1953).
13. Lightfoot, L. H., and Coolidge, T. B., *J. Biol. Chem.* **176,** 477 (1948).

Discussion

ROBERT M. LOLLAR: I think this is a very interesting paper because the literature is all too deficient on information in the variations in any of the properties of collagen which occur in different animals from the same species. I find the comparative results on the three sets of identical twins quite interesting and only wish we had more of such information.

There were certain factors in connection with the work that I thought we might elaborate on a little further. One: in commenting on the differences between the hide powder and the collagen secured from the hides, you noted the difference in the rate of solution. And in the abstract, the term "hydrolysis" is used.

I should like to ask Dr. Hoover if he would care to comment on how far he thinks the breakdown into amino acids has gone during the period of time that the amide nitrogen is being released.

S. R. HOOVER: I believe about half of the free amino nitrogen is released in the six hours. There is not a great deal of free amino nitrogen released in the first hour when the amide nitrogen is complete. It is on the order of five to ten per cent by the time the first hour is up. But then that goes ahead. This is at 70 degrees. At 100 degrees you can get almost complete hydrolysis in six hours.

LOLLAR: That, then, takes me to a part of the next question, which is concerned with the comparative accuracy of the values secured on collagen and the values secured on the other proteins.

First, I note that the level of amide nitrogen is appreciably lower in the case of collagen than it is on the other proteins. We often find that the standard deviation bears some relationship to the magnitude of the value being measured, and I think of that particularly in connection with some work we have done in connection with the free amino nitrogen technique on incompletely hydrolysized proteins where we find the error term of this extrapolated value is much larger where the degree of hydrolysis is small.

I did not see in the slides or the paper, as I had a chance to look over it, any standard deviation which specifically applied to collagen.

HOOVER: I was waiting to see if it was there, too.

The answer is that the standard deviation is essentially the same for the different materials and the reason that is true is that we adjust our sample size so that we get the same amount of ammonia off from the various samples. In the collagen we took three times as much material to run as we did with casein, but that does not cause any complications. So that I do not believe there is reason to expect any significant difference in error in a sample that is low in amide nitrogen. Actually, Mr. Viola usually runs a pilot to decide how much sample he is going to take if he cannot guess fairly well what his answer is going to be.

We try to shoot at about this level of 350 to 500 microliters, half a milliliter, of titration.

LOLLAR: In other words, the standard deviation of an individual value, whether it be on collagen or on one of the other proteins, is essentially the same?

HOOVER: Yes.

LOLLAR: If we then take this information and apply it to some of the differences as, for instance, the difference between the more native collagen and limed collagen (if I can remember the tables correctly, the difference is just roughly six standard deviations) it indicates to me that rather large differences would have to be secured for any small size experiment. How many replicate analyses are represented, for instance, in a comparison between the native collagens and the limed collagen?

HOOVER: Each point is a mean of three results. And in general those three results actually agree within about a tenth of a per cent. Now, I do not think we have pushed this, as I said in my original apologia, we have not pushed this to the limit that can be done for precise comparisons of the type you have in mind. I think the standard deviation can be held to underneath a tenth of a per cent.

LOLLAR: I was interested, for instance, in the three sets of twins, that all three sets of the restricted twins had actually a higher ratio of amide nitrogen. But that is not significant with the error encountered in the experiment at hand except that three coincidences has a certain significance.

LUDWIG SELIGSBERGER: (QM R. & D Command): In a paper in 1937 Kuentzel* mentions that he boiled collagen in NaOH, and determined the insoluble matter which he called elastin at that time. He found, of course, much more in the flesh layer than in the corium. Have you observed anything insoluble in this experiment which you could possibly determine? Would you expect that layerwise analysis would lead to differences in the amino nitrogen? Also, what is your opinion as to a possibility to differentiate between collagen and elastin?

HOOVER: I believe you made three points.

The first is that in this work reported here, we had the middle split. The corium was what we were working on in all cases except the white hide which was a regular white hide. We have done a great deal of work in connection with the purification of collagen. We have not been at it very long but have done a great deal of work in that time, trying to get the differential resistance to alkali on some reasonable basis. Frankly, Dr. Seligsberger, we have not. The collagen-elastin argument is a mystery as far as I am concerned.

Your third point?

SELIGSBERGER: Have you visually observed anything insoluble in these experiments?

HOOVER: No. We were using the corium split and it all goes into solution.

DONALD F. HOLLOWAY: One of those was a lime cured gelatin. Was the other acid cured or lime cured?

HOOVER: The answer is yes. One of each. They are Eastman top grades of gelatin and some of you gentlemen may be familiar with them. They worked out and published it in 1929 for the photographic grades and they have not changed one iota the type of processing.

HOLLOWAY: You have one acid cured and one lime cured and that accounts for the difference in the analysis?

*C 1936, 567.

HOOVER: Yes, but it is more than just liming, to get it down there. After all, with hide power and other things we fooled with, you do not get down to the level of 0.4 per cent for the amide nitrogen. I do not know how they get down to such a value.

HOLLOWAY: It was the acid one that had the low value, didn't it?

HOOVER: They claim the acid treated stuff has the high pH. It has the highest iso-electric point.

HOLLOWAY: The low amide nitrogen?

HOOVER: No, it is the one with the high amide nitrogen.